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This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. The goal of this research was to investigate the effects of the small stress protein, HSP27, on growth and motility characteristics of normal and tumor-derived human mammary cell lines. We hypothesized that cells overexpressing HSP27 would show increased motility, altered chemotactic properties, increased resistance to heat killing and to certain drugs. Donna has prepared and studied 19 clonal MDA231 breast tumor cell lines that overexpress human HSP27, and determined that, while heat resistance is increased in these cells, there is no consistent effect on their proliferation rates, drug resistance, motility or invasiveness. It is probable that the conflicting reports on the effect of increased HSP27 levels on mammary cells reflects clonal variation among the cell lines used. An additional study on *hsp27* gene regulation in mammary cells following estrogen administration suggests that the ensuing increased HSP27 mRNA levels do not reflect a direct response to estrogen acting through the estrogen receptor on the *hsp27* promoter sequences. Donna's Dissertation Committee has requested replication of specific experiments, but agree that she may begin writing. She should defend her thesis in December.

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Gileen Hickey 9/2/99
PI - Signature Date

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INTRODUCTION

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender.

The goal of this research is to investigate the effects of the small stress protein, HSP27, on growth and motility characteristics of normal and tumor derived human mammary cell lines. Preliminary clinical studies indicate that elevated levels of HSP27 in breast tumor cells correlates with aggressive metastasis and poor prognosis (1;2). We have shown that HSP27 overexpression confers resistance to killing by hyperthermia and by certain anti-tumor drugs (3;4). Phosphorylation of HSP27 increases rapidly in cells treated with heat, cytokines or mitogens (5-8). In rodent cells overexpressing human HSP27, the actin cytoskeleton is resistant to damage caused by hyperthermia or cytochalasin D treatment (9;10). High levels of HSP27 also correlate with increased accumulation of cortical actin, suggesting a possible effect on cellular motility. In contrast, cells expressing a non-phosphorylatable form of HSP27 show inhibition of processes depending on cortical microfilament dynamics (10).

Our study is based on the hypothesis that HSP27 is a component of a signal transduction pathway that regulates actin microfilament dynamics, and may affect cell migration and the metastatic potential of tumors. We propose that cells overexpressing HSP27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and to certain drugs. Overexpressing cells may respond more vigorously to chemotactic agents, or may respond to different signaling molecules than the parent cell type. We predict that the cells expressing antisense HSP27 sequences, or those expressing the unphosphorylatable mutant will show responses antagonistic to those shown by cells overexpressing normal HSP27.

We proposed to 1) prepare human mammary cell lines expressing either increased levels of HSP27, unphosphorylatable HSP27, or antisense sequences that reduce endogenous HSP27 expression; 2) assay the rate of cell proliferation in these cell lines, compared to controls; 3) assay motility and response to cytokines of these cells using the Boyden chamber technique; and 4) study the resistance of the cell lines to hyperthermia, arsenate, cytochalasin D, and antitumor drugs.

PROGRESS REPORT

PROGRESS REPORT

I. ACADEMIC:

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. Donna has completed all required academic course credits for her degree. She has had her penultimate Dissertation Committee Meeting, which took place on August 5, 1999. The decision of the committee was that she had essentially completed the work needed for a dissertation, and may begin writing. Her research has been complicated by the fact that many studies yielded negative results, and these results contradict some published studies. These results nonetheless were consistent, and clarify the basis of some of the controversy in the literature about the role of the small heat shock protein in tumor cells. Her studies are therefore important. At the same time, her committee felt it necessary that a limited number of specific experiments be replicated again, to firmly establish the validity of her results. These experiments are indicated in the Future Studies section of the body of the report. They are underway, and Donna is expected to have her Dissertation completed and defended by the end of this semester.

II. RESEARCH:

These predoctoral studies were directed towards understanding how HSP27 protein content in human mammary tumor cells affects parameters of cell growth and survival. The working hypothesis was that modulation of HSP27 content and phosphorylation in mammary cells will influence the dynamics of the actin cytoskeleton, leading to changes in proliferation rate, motility, and responsiveness to growth factors. To test this Donna proposed to study the effect(s) of HSP27 content on parameters relating to tumorigenic properties.

The proposed approach was the following:

1. Plasmids will be constructed that allow expression of the *hsp27* gene independently of estrogen under control of a metallotheionine promoter in human mammary cell lines. Constructions expressing normal *hsp27*, non-phosphorylatable mutants of *hsp27*, and antisense sequences for down-regulation of *hsp27* expression will be prepared.

2. Stable cell lines will be developed in ER negative MDA-231 cells in which HSP27 expression can be induced independently of estrogen using a metallothionein promoter. Stable cell lines will be selected in ER+ MCF7 cells in which HSP27 expression can be downregulated by antisense gene constructions.

3. Cells expressing different levels of HSP27 will be compared with respect to: a. the rate of cell proliferation; b. levels of motility and response to cytokines, using a Boyden chamber assay; and c. resistance to hyperthermia, arsenate, cytochalasin D, and antitumor drugs.

4. The importance of HSP27 phosphorylation for the effects observed in Aim 3 will be studied by transforming MDA-231 cells with genes encoding non-phosphorylatable variants of the *hsp27* gene.

A. Development of clonal cell lines overexpressing HSP27:

The construction of expression plasmids for overexpression and antisense repression of HSP27 in transfected cells was accomplished in the first year of the fellowship. The process of transformation and selection of sufficient numbers of stable cell lines for the proposed studies was carried out over a long period of time, much of which included time waiting for the colonies to grow. During this time, Donna initiated studies to determine the mechanism by which estrogen treatment brings about increased levels of *hsp27* mRNA. These studies were not included in the original Statement of Work, but have been reported in previous Progress Reports, and were the basis of Donna's presentation at the Era of Hope Meeting in 1997. The results of these studies comprise part of her Dissertation. The last section of this report summarizes the studies on *hsp27* gene regulation.

Stable cell lines overexpressing HSP27 were created in MDA-231 breast tumor cells, a line usually expressing very low levels of this protein. Cells were selected for puromycin resistance after cotransfection with the antibiotic resistance plasmid, pPUR, and one of two expression plasmids, SV27 containing the *hsp27* gene driven by the SV40 promoter, or Hβ27 containing the *hsp27* cDNA driven by the β-actin promoter. Eight and 11 clonal cell lines were isolated from cells transfected with SV27 or Hβ27, respectively. Immunoblot analyses using anti-human HSP27 (StressGen) showed that the SV27 clonal lines express 7-10 fold more HSP27 than the parental control, and the Hβ27 clones express only 2-4 fold more HSP27 than the parental line (Fig. 1). The SV27 clonal cell lines express HSP27 at levels similar to those seen in the ER positive MCF7 cell line. These clonal cell lines were used to study the effects of HSP27 overexpression on thermoresistance, proliferation rate, drug resistance, and motility.

Stable cell lines expressing antisense *hsp27* gene sequences were not successfully isolated. Attempts to use the adenovirus vector system to express antisense *hsp27* sequences in mammary tumor cells were described in the 1997 Progress Report, and were unsuccessful. The studies with overexpression on HSP27 in mammary tumor cells showed little phenotype, as described below, and this diminished the probability that antisense studies would be productive. This line of research was not followed further.

B. Effect of overexpression of HSP27 on stress resistence and tumor-related phenotype of breast tumor cells:

a. Thermoresistance of HSP27 overexpressing cell lines

In order to determine if the HSP27 overexpressed in these clonal lines was functional, each cell line was examined for resistance to heat killing by exposure to 44°C for one to four hours. Cells were allowed to recover for 10 days, and surviving cells were stained. Survival was determined by observing the density of the stained cells. MDA-231 parental controls and vector transfected controls (KS) all died by 2 hours at 44°C. However all HSP27 overexpressing cell lines showed a significant increase in heat resistance comparable to that of MCF7 cells which naturally overexpress high levels of HSP27. The majority of cells are still alive at 2 hours and some cells still survive after 4 hours of heat treatment (Fig. 2). To more precisely quantitate the resistance to heat killing, a clonal survival experiment was done on a single clone, SV27-18, representing one of the high level overexpressors. Its thermoresistance was compared to that of the parental line and a vector control (Fig. 3). After two hours at 44°C, only 0.001% of control cells survived. Pre-stressing cells at 44°C for 20 minutes followed by 16 hours of recovery induces all of the stress proteins and confers a state of classic thermotolerance, resulting in a survival of 10% of the cells after four hours at 44°C. Thermoresistance in the HSP27 overexpressing cell line approached that conferred by a state of thermotolerance, resulting in 30% survival at 2 hours and survival of 0.5% of the cells after four hours at 44°C. These results confirm that the HSP27 overexpressed in the clonal cell lines affects the cell phenotype and confers more than 600-fold increase in protection from heat killing.

b. Proliferation rate of HSP27 overexpressing clonal cell lines

HSP27 overexpressing clonal cell lines plated in 12-well tissue culture plates were manually counted daily, and doubling times were calculated (Fig. 4). The average doubling times of parental or vector-transfected controls were 28 to 35 hours, respectively. Two HSP27 clones appeared to have a slower growth rate, but were not statistically different from the controls. The average doubling times of the remaining HSP27 overexpressing clonal lines and those of pooled transformants were comparable to controls, demonstrating that increased expression of HSP27 in MDA-231 cells has no significant effect on proliferation rate. These results conflict with previous mutually contradictory reports on MDA-231 cells expressing increased levels of HSP27. In one study, pooled transformants showed a decrease (11), and in another, clonal cell lines showed an increase in proliferation rate (12). However the published studies have presented results from a single pooled (11) or stably transfected (12) cell line. Our analysis is based on the results from 18 different clonal cell lines, and suggests that many of the observed differences are due to clonal effects and may not be due specifically to HSP27 expression. Currently, experiments are underway to repeat and further validate our conclusion.

c. Drug resistance of HSP27 overexpressing clonal cell lines

In order to determine if HSP27 overexpression affects resistance to toxic agents in breast tumor cell lines, the clonal lines were exposed to two chemotherapeutic drugs, doxorubicin and cisplatin, as well as to hydrogen peroxide which induces oxidative stress, and sodium arsenite, which induces protein aggregation. Clonal lines were exposed to five different concentrations of each substance for one hour, then trypsinized and replated at known concentrations. Cells were allowed to recover for 10 days in drug-free media, and surviving colonies were stained and counted. The concentration of each substance resulting in 50% survival (IC50) was calculated (Fig. 5). Comparison of the IC50 of control cells to that of the clonal lines shows no consistent or significant differences in survival of cells overexpressing HSP27, demonstrating that HSP27 has no effect on resistance to doxorubicin, cisplatin, H_2O_2, or sodium arsenite in these clonal cell lines. These data again support the hypothesis that clonal variation may account for the previously reported results (3;12-21), which used a limited number of cell lines to show that HSP27 confers resistance to some drugs.

d. Effect of HSP27 overexpression on motility and invasiveness of clonal cell lines

To determine if high levels of HSP27 affect the rate of migration, we compared migration of clonal cell lines to that of the parental or vector-transfected controls. Cells were placed in the upper compartment of a modified Boyden chamber, and allowed to migrate through a membrane to the bottom chamber. The bottom chamber contained media with or without a 1% fetal calf serum (FCS), which we used as general chemoattractant. After four hours, cells that migrated through the membrane were stained and counted. Basal migration is represented by the number of cells that migrated in the absence of serum. Although the HSP27 overexpressing cell lines differ somewhat from the control cell line, there was no consistent difference in basal motility in either the high or low level overexpressors (Fig. 6 & 7). The low-level overexpressors showed no consistent difference in the number of cells migrating toward the chemoattractant (Fig. 6). However motility toward 1% FCS significantly decreased in high level overexpressors compared to the parental control (Fig. 7). This difference may be due to the increased expression of HSP27, however there may be other factors affecting the motility of these cells. Again clonal variation may be a major factor affecting the outcome of these studies, as can be seen from the differences between clonal cell lines transfected with an empty vector which do not overexpress HSP27 (Fig. 8). Currently studies are underway to replicate and verify the results.

The invasive potential of the clonal breast tumor cell lines was determined by measuring their ability to move through Matrigel, a substance which mimics the extracellular matrix. HSP27 overexpression resulted in decreased invasiveness in most clonal lines tested compared to controls (Fig. 9), although one low level overexpressor (Hβ19) showed an increased invasive activity. The pattern of invasion through the membrane in the high level overexpressors resembles that of their motility. But the clonal variability seen in the two low-level overexpressors indicates that invasiveness is influenced by other factors besides HSP27.

Our results consistently show variability in the tumor-related phenotype of clonal cell lines overexpressing HSP27. The characteristics of the clonal cell lines do not vary proportionately to HSP27 levels. They also do not differ in any consistent direction from that of the parental cell line or of vector-transfected controls, except in the case of heat resistance. The overexpressing cells are consistently manyfold more heat resistant than the cell lines lacking HSP27 expression, as has been reported in numerous cell types (4;22-24). Our conclusion is that the contradictory observations reported in the literature reflect clonal variation among the cell lines used for those studies.

C. Studies of the effects of estrogen on *hsp27* gene expression:

Estrogen treatment of mammary tumor cells has been reported to be followed by increased levels of HSP27 (25-28). Considerable periods of time were available during selection and colony growth of clonal cell lines for the experiments described above. We used this time to undertake an investigation of the mechanisms underlying the estrogen induced change in *hsp27* mRNA levels in mammary cells.

Estrogen responsive genes typically contain a palindrome of the consensus sequence 5'-GGTCAnnnTGACC-3', to which the estrogen receptor (ER) binds as a homodimer (29). Binding of estrogen to the ER is thought to trigger a conformational change in the ER, which then activates transcription (30). Estrogen responsive genes have been identified which contain several half-palindromic sequences within their flanking regions (31-36) (37-39), referred to as ERE-half sites, which have been shown in some cases to be important in estrogen regulation of gene activity (31;34-37). The *hsp27* promoter has two perfect ERE-half sites within its upstream sequence at -92 and -925. Additionally there is an imperfect ERE-half site at -74 (Fig. 10). The two half-EREs at -74 and -92 form an imperfect ERE with an intervening sequence containing a TATA box. Any or all of these elements may play a role in estrogen induced transactivation. The *hsp27* promoter also contains regulatory elements such as AP2 (-146), three SP1 sites (-108, -151, -1007), two TATA boxes (-32, -83), and a CAAT box (-376). Additionally the *hsp27* promoter contains a heat shock element (HSE) at position -185. This regulatory sequence is conserved in heat shock gene promoters and is bound by the heat shock factor (HSF), a transcription factor which is activated in response to elevated temperatures, and controls heat induced transcription from heat shock gene promoters.

a. <u>Construction of reporter gene plasmids</u>

The experimental strategy for analyzing the interactions between the ER and the *hsp27* promoter involved using reporter gene analysis to assay for promoter activity. Two primary plasmids were constructed (Fig. 11), one in which 1091 bp of upstream sequence from the *hsp27* promoter drives the firefly luciferase gene, this is referred to as the promoter only construction, Bg27Lux. In the second plasmid, the luciferase gene was placed within the first exon of the *hsp27* gene, and is driven by the same promoter region. This whole gene construction, Bg27EXLux, allows for the presence of any proposed regulatory elements in the downstream sequence, one of which is an ERE-half site within the first intron. Plasmids were also created containing truncated promoter regions to determine how much of the upstream sequence is needed for transcriptional activation, and are named for the restriction enzyme with which they were created, Bm27Lux, Pst27Lux, and Eco27Lux (Fig. 12). These plasmids were transfected into cells which were subsequently stimulated with heat or estrogen. Levels of transcriptional activation were determined by measuring luciferase activity in cell lysates.

b. <u>Effect of heat on activity from the truncated *hsp27* promoter plasmids</u>

To test whether the promoter plasmids were functional, heat inducibility of the promoter truncations was measured in ER positive and negative breast tumor cell lines. The full length or truncated promoter plasmids were transfected into either ER positive MCF7 cells, or ER negative MDA-231 cells, which were heat treated for 1 hour at 42°C and recovered at 37°C for 3 hours, or left untreated (Fig. 13). Luciferase activity from cell lysates showed that most of the basal and heat induced activity resides within the proximal 216 base pairs. Additionally, presence of the HSE is necessary for heat induced transcription, but does not affect basal transcription. The presence of the ER is not necessary for heat induced transcription because the promoter was still inducible in MDA-231 cells. These results also confirmed that this system is functional for assessing promoter activity.

c: <u>Estrogen responsiveness of the *hsp27* promoter</u>

The effect of estrogen on transcriptional activation of the *hsp27* promoter was assessed by transfecting the full-length promoter plasmid into estrogen deprived MCF7 cells. Luciferase activity was measured from untreated cells or those treated with either heat or 10^{-7} M estrogen (Fig. 14). Heat treatment induced luciferase activity within 2 hours. Activity peaked at 4 hours and returned to background levels by 24 hours. However promoter activity from cells treated with 10^{-7} M estrogen, which we found optimal for induction of endogenous *hsp27* mRNA (Fig. 18), did not differ from non-treated controls.

Because the promoter showed no response to estrogen, we first assayed whether the cells still contained a functional ER. Activity from a reporter plasmid containing a perfect palindromic ERE driving the luciferase gene, ERELux, was compared to that of the full-length *hsp27* promoter, Bg27EXLux. These plasmids were transfected into estrogen deprived MCF7 cells and measured for estrogen-stimulated activity over time (Fig. 15). The ERE was immediately responsive to estrogen with activity peaking between 4 and 8 hours, demonstrating that these MCF7 cells contain a functional ER and the system was responsive to estrogen. However the *hsp27* promoter remained unaffected by estrogen, indicating that estrogen does not affect transcriptional activation from this sequence.

The role of ER in *hsp27* gene activation was tested using a second system. Two ER negative cell lines, HeLa and MDA-231, were cotransfected with a gene encoding a mutant constitutively active ER along with the *hsp27* promoter plasmid, Bg27Lux, or ERELux (Fig. 16). Basal transcription in the presence of normal media and serum was measured in the presence or absence of transiently expressed ER. In both cell lines activity from the ERE increases 2-3 fold in the presence of transiently expressed ER, however the *hsp27* promoter remained unaffected by the presence of an ER. These results are consistent with our previous results showing that the *hsp27* promoter sequence is not responsive to estrogen or ER.

It has been reported that in order to show estrogen responsiveness in MCF7 cells, it is necessary to transiently transfect additional ER (40;41). Because the experiments in MCF7 cells had not involved cotransfection of ER with the reporter plasmid, it was necessary to perform another experiment to confirm the lack of response of the *hsp27* promoter to estrogen. To test the effects of transiently expressed ER on expression from the *hsp27* promoter in MCF7 cells, the constitutively active ER was cotransfected into estrogen-deprived MCF7 cells along with ERELux, Bg27Lux, or Eco27Lux. As seen in the previous studies, the promoter containing an ERE showed a 10 to 12 fold increase in activity after addition of estrogen, however both full length (1091 bp) and short (216 bp) *hsp27* promoters remained unaffected by estrogen (Fig. 17). Interestingly, expression from the ERE is consistently less in the presence of additional ER in the MCF7 cell line. These observations are in contrast to previous reports indicating the need for transiently expressed ER to see estrogen responsiveness in this cell line.

These data consistently show that there is no effect by estrogen or the estrogen receptor on transcriptional induction from 1091 bp of upstream sequence of the *hsp27* promoter. One possible explanation for these findings is that regulatory elements exist distal to the gene segment studied. The 5'-flanking sequence used in these experiments was chosen because it contained two perfect and one imperfect half-EREs in a context suggesting possible regulation by estrogen. A search of the sequences available in the databases upstream of those we used does not reveal any more recognizable estrogen-responsive sequences. The most likely conclusion is that the estrogen/ER complex does not directly induce transcription. This is supported by recent studies suggesting that the ER plays an indirect role in induction from the *hsp27* promoter by interaction with Sp1 (41;42), a nuclear transcription factor which induces transcription via interaction with regulatory sequences found in many gene promoters. A novel transcription factor, HET, was recently cloned which binds the *hsp27* promoter at the imperfect ERE containing a TATA element in the spacer region (-74 to -92) (43). This protein was shown to have a repressive effect on basal expression from the *hsp27* promoter, although the effect of estrogen on activity from this transcription factor was not investigated.

d. Investigations of mechanisms underlying estrogen influences on *hsp27* mRNA levels

Evidence from the above studies support our finding that the ER is not the main factor responsible for the increased levels of *hsp27* transcription after addition of estrogen. Nonetheless, *hsp27* mRNA levels do increase in response to estrogen treatment (Figs. 18) (26-28). We undertook to determine if estrogen-stimulated increases in *hsp27* mRNA are an indirect result of general effects of estrogen on cell cycle or mRNA stability.

The first question addressed was if the increase in estrogen-stimulated *hsp27* mRNA levels was due to an increase in transcription of the gene. A nuclear run-off assay was used to measure the relative rates of transcription over time after estrogen treatment. In order to investigate the effects of estrogen on the half-life of the *hsp27* mRNA, actinomycin D was used to block transcription and then the amounts of *hsp27* mRNA remaining after treatment in the presence and absence of estrogen were determined.

Transcription was measured using a nuclear run-off assay, in which nuclei were isolated from MCF7 cells at various times after estrogen treatment. Nascent mRNA was then elongated in vitro in the presence of radiolabelled nucleotides and then hybridized to membranes containing several cDNAs representing *hsp27*, *hsp70* or the proliferation dependent transcripts for *histone H4* (44;45), and *hsp90* (46), and the elongation factor *EF2* (47;48). *EF2* was chosen as a housekeeping gene control, but we now find that it, too, is proliferation responsive. Although the blot is not very pretty, it shows that transcription of *hsp27* mRNA increases over 24 hours (Fig. 19). We also see an increase in levels of transcription of several proliferation responsive mRNAs, especially *histone H4*. This suggests the possibility that the increase in transcription of *hsp27* mRNA may be due to a general effect on proliferation by estrogen, and is not a direct effect of the estrogen/ER complex on regulatory elements within the promoter region. This assay will be replicated with a proliferation independent gene to be selected to use as negative control.

Estrogen has been shown to have stabilizing effects on several mRNAs (49-52) (53;54). Therefore it is possible that although *hsp27* mRNA increases at the transcriptional level following estrogen treatment of cells, some of the observed increases in mRNA levels may be due to an RNA stabilizing effect influenced by estrogen. To test this hypothesis, the stability of *hsp27* mRNA was determined in the presence and absence of estrogen. MCF7 cells deprived of, or stimulated with, estrogen were treated with the transcriptional inhibitor, actinomycin D (AD). Levels of *hsp27* mRNA were measured over time after AD treatment, and the half-life of the mRNA was measured. The *hsp27* mRNA was found to have a relatively long half-life of 29.8 hours, and stability was not significantly affected by the presence of estrogen (Fig. 20).

Several factors have made analysis of estrogen influence on *hsp27* mRNA levels difficult. First, the *hsp27* mRNA is only induced by approximately two-fold in response to estrogen, and constitutive levels are very high, which makes determining differences in levels difficult. Additionally, the half-life of *hsp27* mRNA is approximately 30 hours, therefore any change in mRNA levels tend to be masked by mRNA already present. However, from the data obtained, it appears that the increase in *hsp27* mRNA levels in response to estrogen may be due to a general proliferative response to estrogen, and not to a direct effect on transcriptional induction via regulatory elements located within the *hsp27* promoter.

Future studies

Several experiments are being repeated to confirm the result that overexpression of *hsp27* does not confer tumor related phenotypic traits and resistance to drug treatment on breast tumor cells. In particular, the proliferation experiments, the doxorubicin resistance studies, and the migration studies will be replicated again. In addition, the nuclear run-off study of transcriptional activation following estrogen administration will be repeated. Donna is beginning to write her literature review, and should defend her Dissertation this winter.

Key Research Accomplishments

- As this research was undertaken as a predoctoral fellowship, a major accomplishment has been the development of the ability of the student, Donna, to originate, plan and execute an independent research project, including troubleshooting, acquiring and introducing new techniques to approach her research goals, and initiating collaborative approaches to help her solve research problems.
- The contradictory results published concerning the effects of *hsp27* expression in tumor cells may only reflect the variability inherent among selected clonal cell lines.
- Estrogen related increases of *hsp27* mRNA and protein do not reflect direct induction of transcription from the *hsp27* promoter by estrogen acting through ER binding.

Reportable outcomes

1. Publications:

Abstracts:

Hickey, E., Latour, D., Egender, D.J., and L.A. Weber, The Importance of Phosphorylation for HSP27 Function. Meeting on "Molecular Chaperones and the Heat Shock Response", Cold Spring Harbor, May 1996.

Egender, D.J., Weber, L. A., and E. Hickey, Regulation of the Small Heat Shock Protein Gene in Mammary Tumor Cell Lines DOD Breast Cancer Research Program: "An Era of Hope" Meeting, October, 1997

Publications (in preparation):

Egender, D.J., Weber, L.A., Borrelli, M., and Hickey, E. Influence of HSP27 Levels on Drug Resistance in Mammary Tumor Cells.

Egender, D.J., Weber, L.A., and Hickey, E. Regulation of the *hsp27* gene in mammary tumor cells.

2. Degrees Obtained:

Donna J. Egender Housley: Ph.D. (Expected Dec. 99)

3. Plasmids and Cell Lines:

Plasmids: *hsp27* promoter/luciferase expression plasmids
HSP27 expression plasmids: *hsp27*, SV40, or β-actin promoter driven
Adenovirus/HSP27 expression system.

Cell lines: Stable HSP27 expressing clonal cell lines in MDA-231 cells.

4. Employment/research opportunities applied for:

Donna so far has interviews at two institutions for potential post-doctoral research.

Conclusions

1. Effects of HSP27 overexpression on the phenotype of the MDA-231 cell line:

Overexpression of HSP27 provides thermoresistance at a level approaching that conferred by a state of classical thermotolerance. However, overexpression of HSP27 does not affect proliferation rate, does not increase resistance to doxorubicin, cisplatin, H_2O_2, or sodium arsenite, and does not increase motility or invasiveness. High levels of HSP27 may actually decrease motility and invasiveness when expressed at levels equivalent to those found in the MCF7 cell line.

2. Estrogen effects on *hsp27* gene regulation:

The proximal 216 bp is sufficient for heat induced and basal transcription from the *hsp27* promoter, and the HSE is necessary for heat induced transcription from the *hsp27* promoter. Estrogen administration is followed by increased levels of *hsp27* mRNA over 24 hours, and an increase in transcription of the *hsp27* gene, as well as of several proliferation responsive genes. The 1091 bp of upstream sequence tested do not mediate estrogen induced increases in transcription. Downstream regulatory elements within the *hsp27* transcribed region can not be demonstrated to be important for estrogen induced transcription. Estrogen responsiveness of the 1091 bp promoter could not be stimulated by addition of an ER to ER positive or negative cell lines. Estrogen does not significantly affect the half-life of the *hsp27* mRNA.

3. Significance:

Negative results are not what one would wish as the outcome of long, careful, and persevering research. The importance of these results, however, lies in the clarification they offer to controversial reports in the literature. The results have been thoroughly evaluated and replicated, and we have confidence in their accuracy. They contradict a generally accepted conclusion about the effect of elevated levels of HSP27 in tumor cells. If the currently accepted conclusion is based, in fact, on an artifact of clonal variation, a general direction of research may be misguided.

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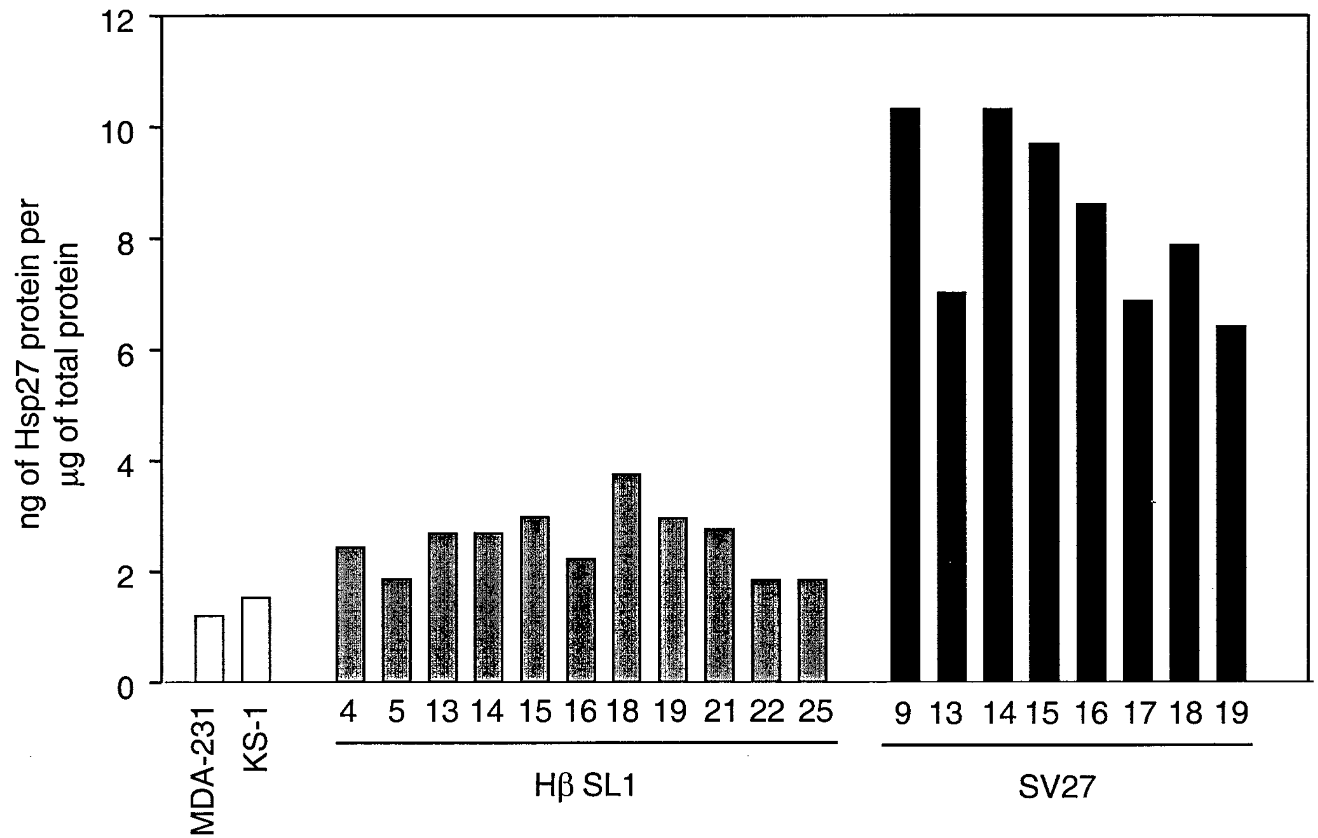

Fig. 1. *Expression of Hsp27 in stably transfected MDA-231 cell lines.* MDA-231 clonal cell lines were examined for expression of Hsp27 by Western blot, and expression was quantitated by phosphoimager analysis of the membrane. Expression in each clonal cell line is shown as ng of Hsp27 protein per μg of total protein.

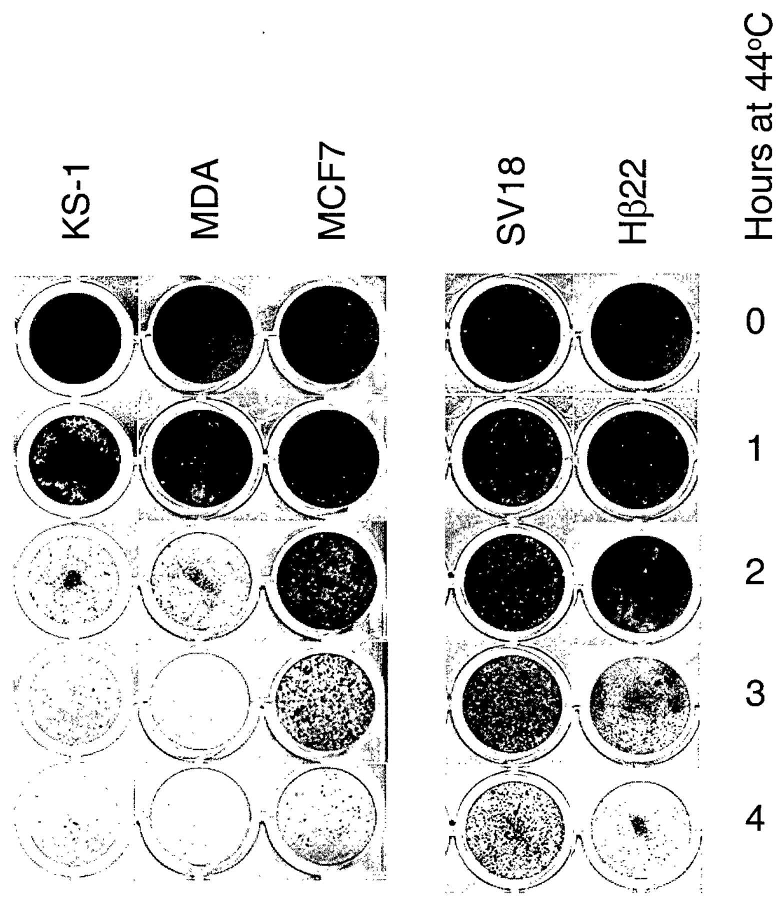

Fig. 2. *Heat resistance of MDA-231, MCF7, and MDA-231 clonal cell lines.* Cells were plated in 24-well plates, and after 24 hours, were heat treated for the indicated times. Cells recovered for 10 days at 37°C, then were stained with Coomasie blue for visualization of surviving cells. The figure shows a representative subset of cell lines; MDA-231, MCF7, KS-1, SV27-18, and Hβ27-22.

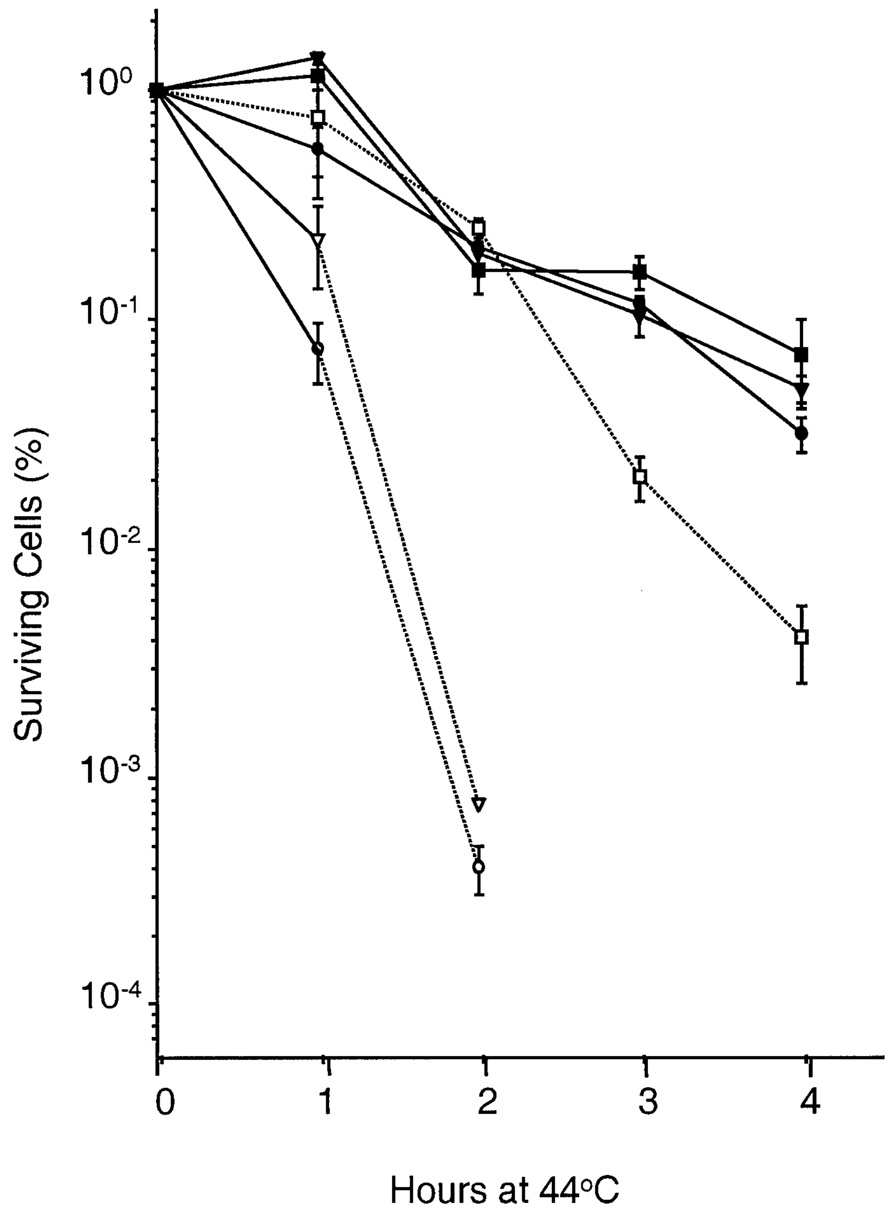

Fig. 3. *Clonal survival following heat stress of untreated and thermotolerant cell lines.* Clonal survival after a heat shock of 44°C for the indicated times is shown for several cell lines: the parental control MDA-231 (···o···), vector-transfectd control KS-1 (···▽···), and the Hsp27 overexpressing clonal line SV27-18 (···□···). Cells of each type, which had been made thermotolerant (TT) by a previous mild heat treatment, were also assayed: TT MDA-231 (—●—), TT KS-1 (—▼—), and TT SV27-18 (—■—). Surviving colonies were stained and counted, and the mean number of surviving colonies ± S.D. was plotted on a log scale.

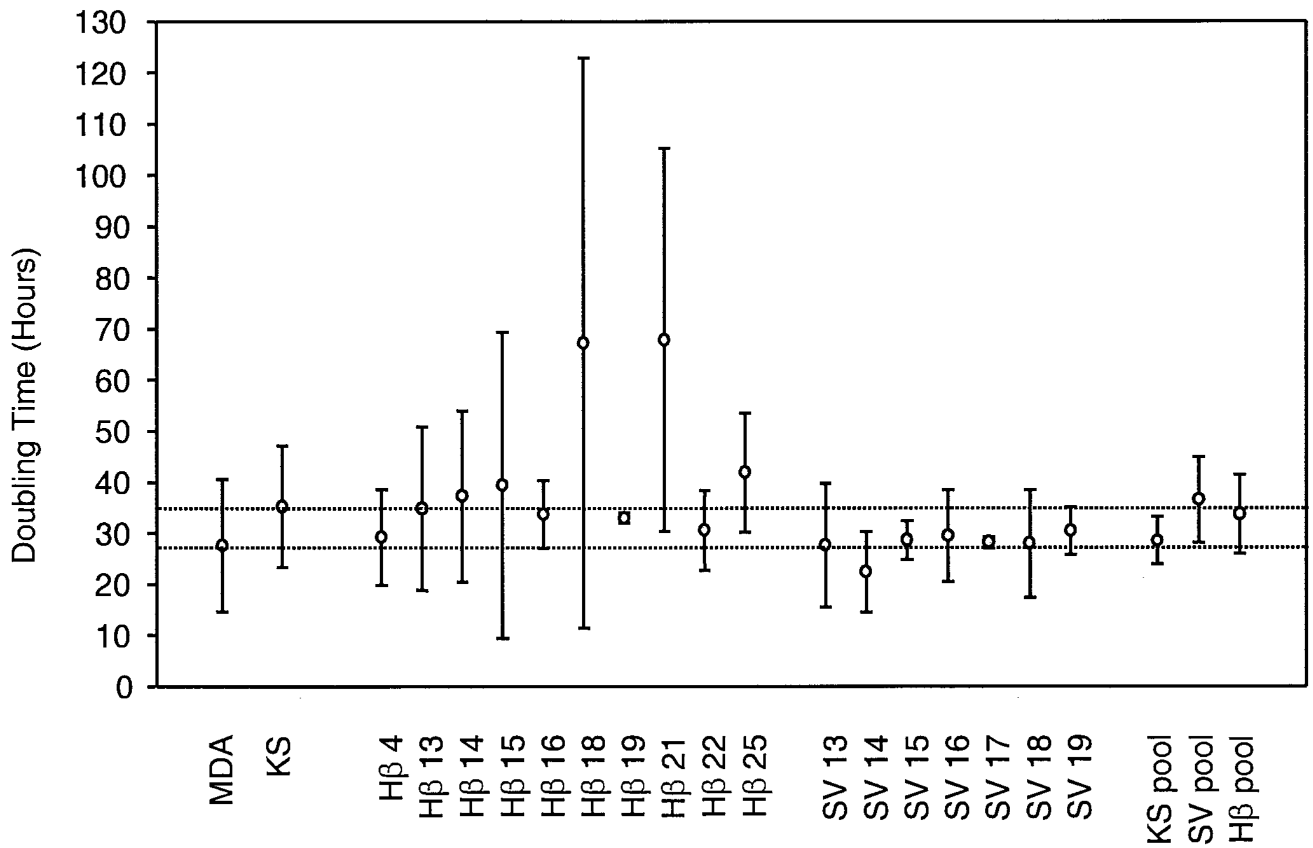

Fig. 4. *Proliferation rate of clonal cell lines overexpressing Hsp27.* Clonal cell lines were plated into 12-well plates and counted daily. Doubling times (in hours) were calculated and the averages were plotted. The two dotted lines represent the average doubling time of the parental control and the KS-1 control.

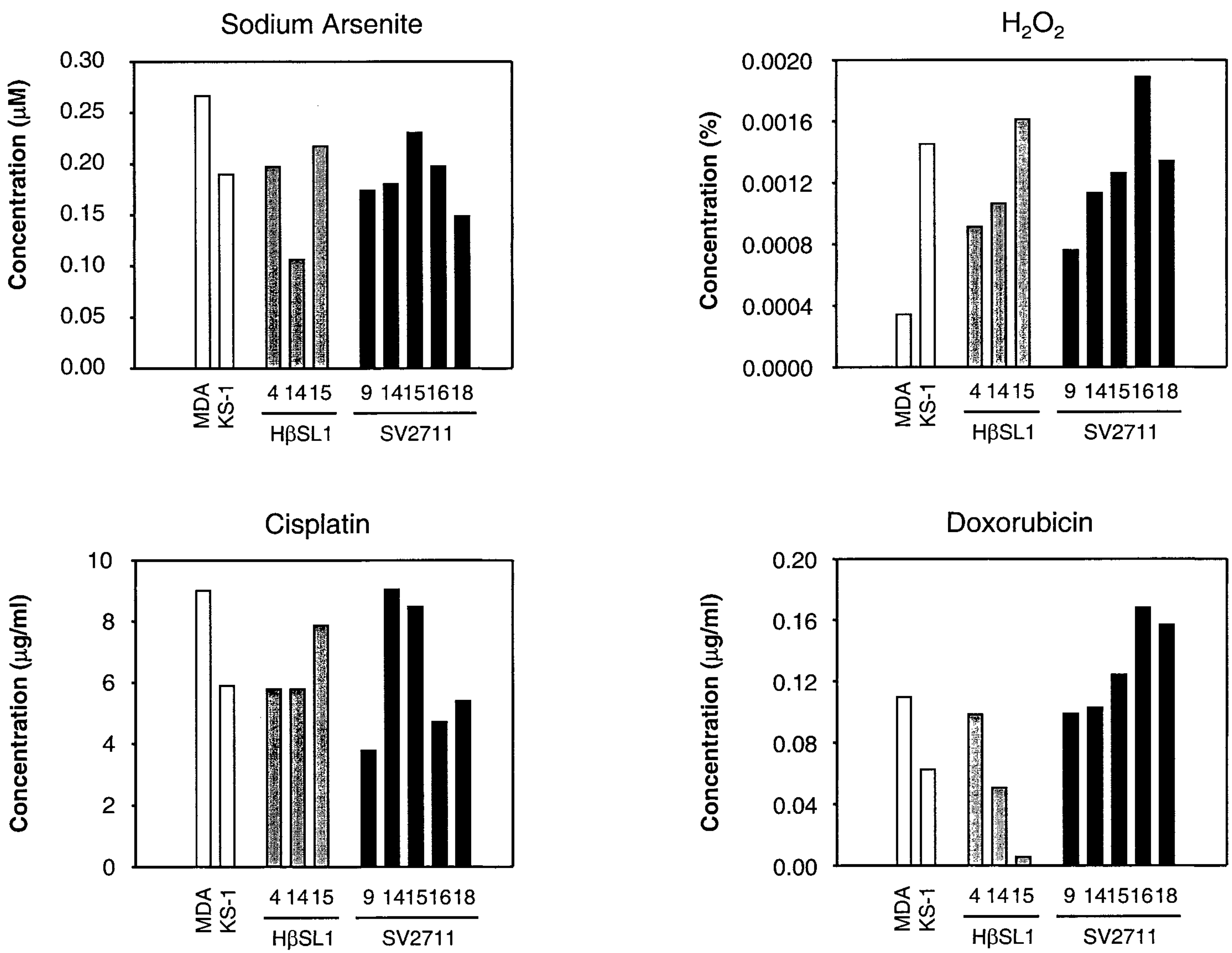

Fig. 5. *IC50 of clonal cell lines.* Cell lines were exposed to the indicated substance for one hour, trypsinized, and replated at known cell numbers. Surviving colonies were counted, and the concentration of drug resulting in 50% survival was calculated and plotted.

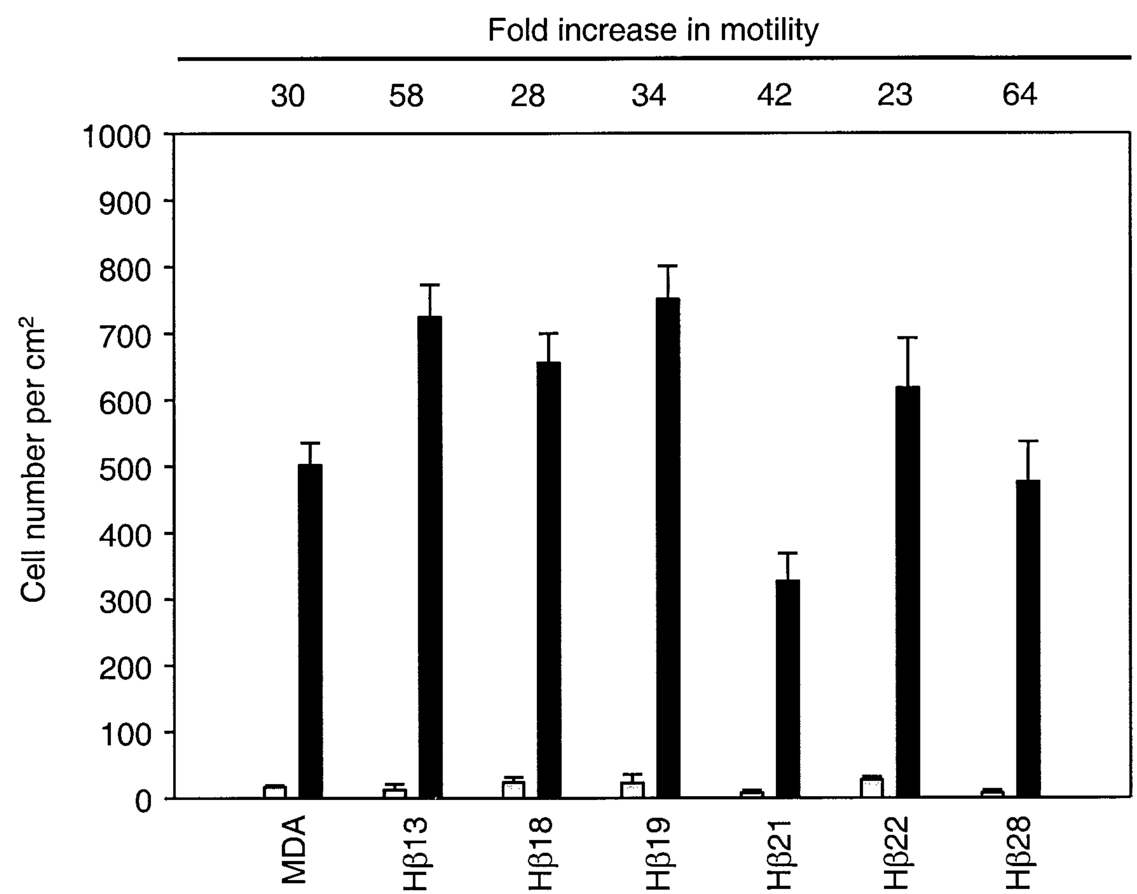

Fig. 6. *Motility of Hβ27 clonal cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (☐) or 1% FCS (■). The fold increase in motility due to attractant is indicated above the graph.

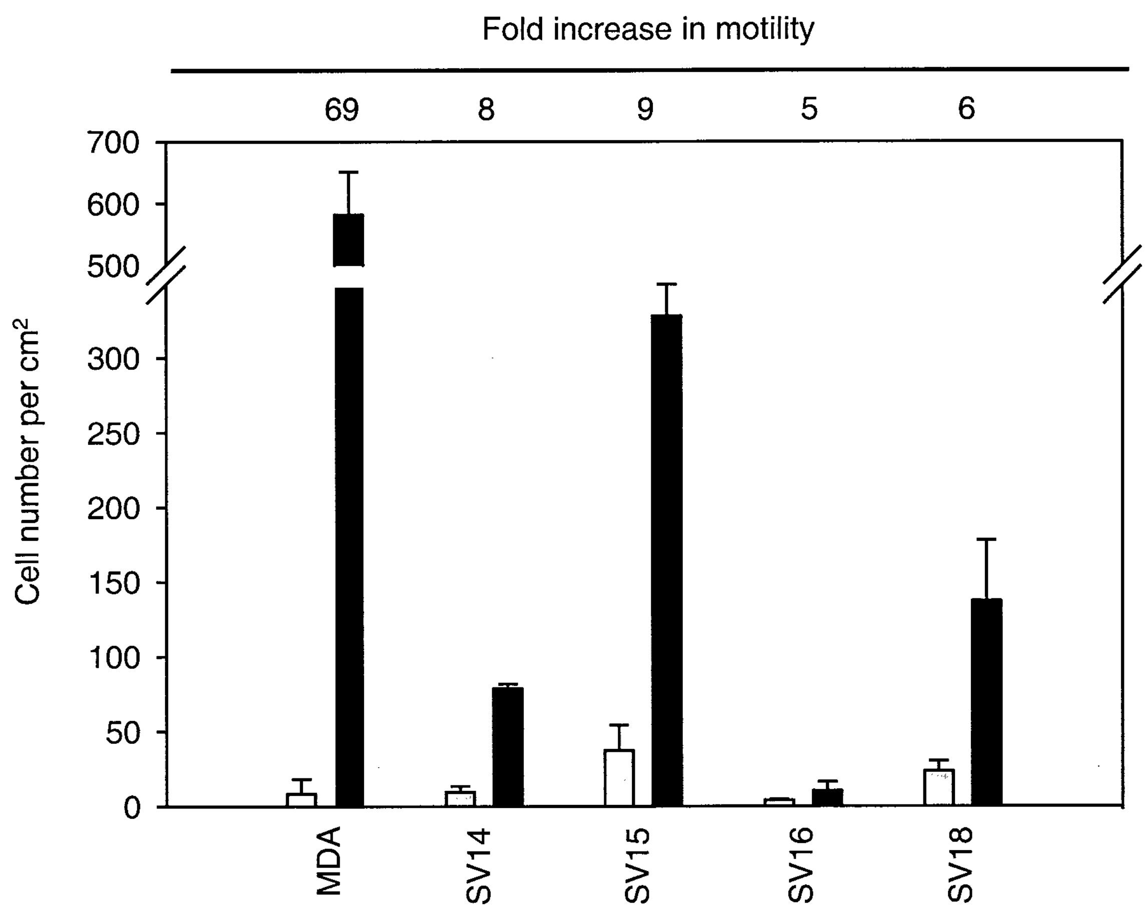

Fig. 7. *Motility of SV27 clonal cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (▭) or 1% FCS (■). The fold increase in motility due to attractant is indicated above the graph.

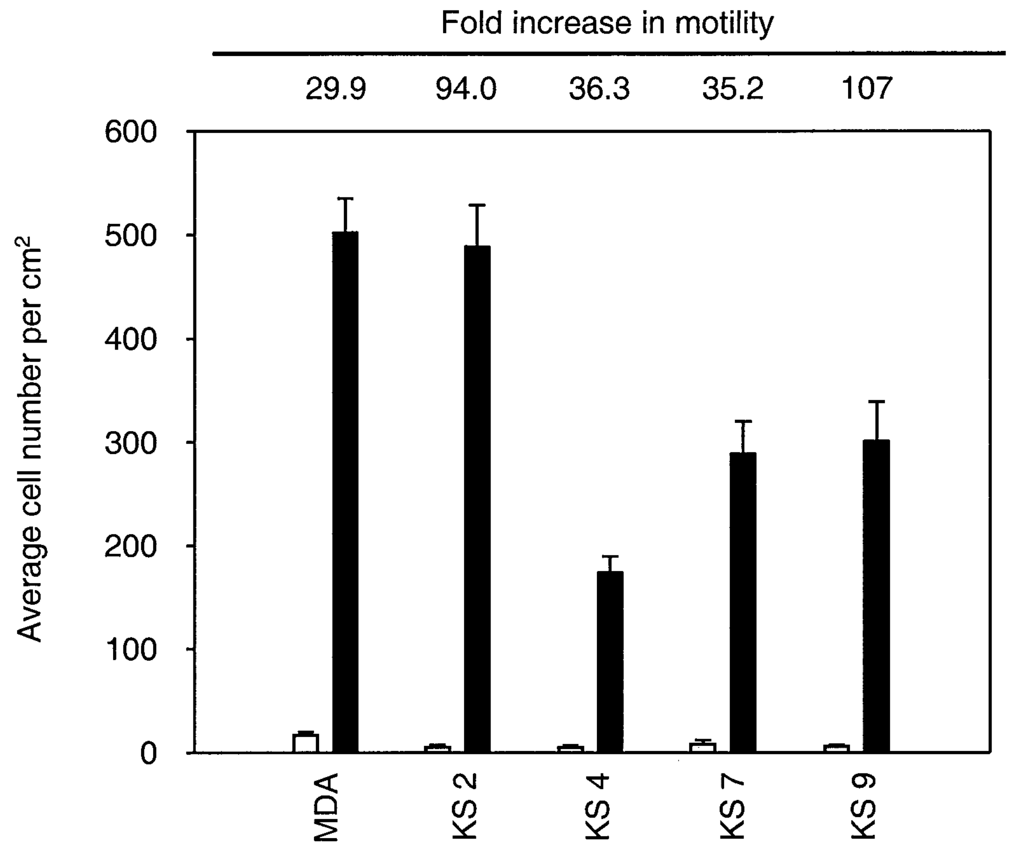

Fig. 8. *Motility of KS clonal cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (▭) or 1% FCS (▬). The fold increase in motility due to attractant is indicated above the graph.

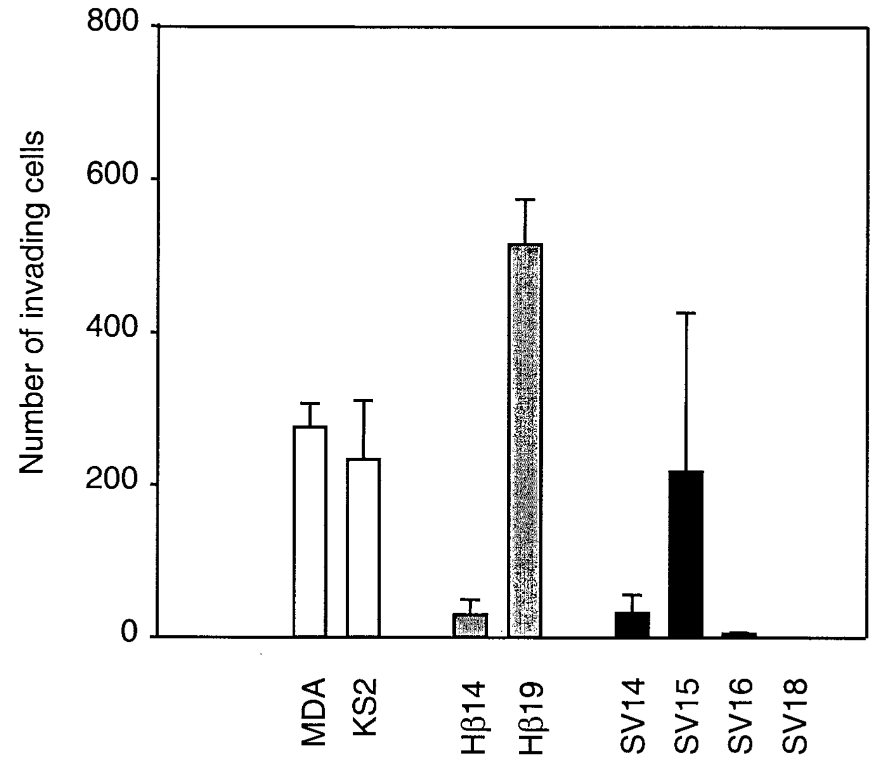

Fig. 9. *Matrigel invasion by clonal cell lines.* Invasion of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the total cells counted per transwell in response to 1% FCS.

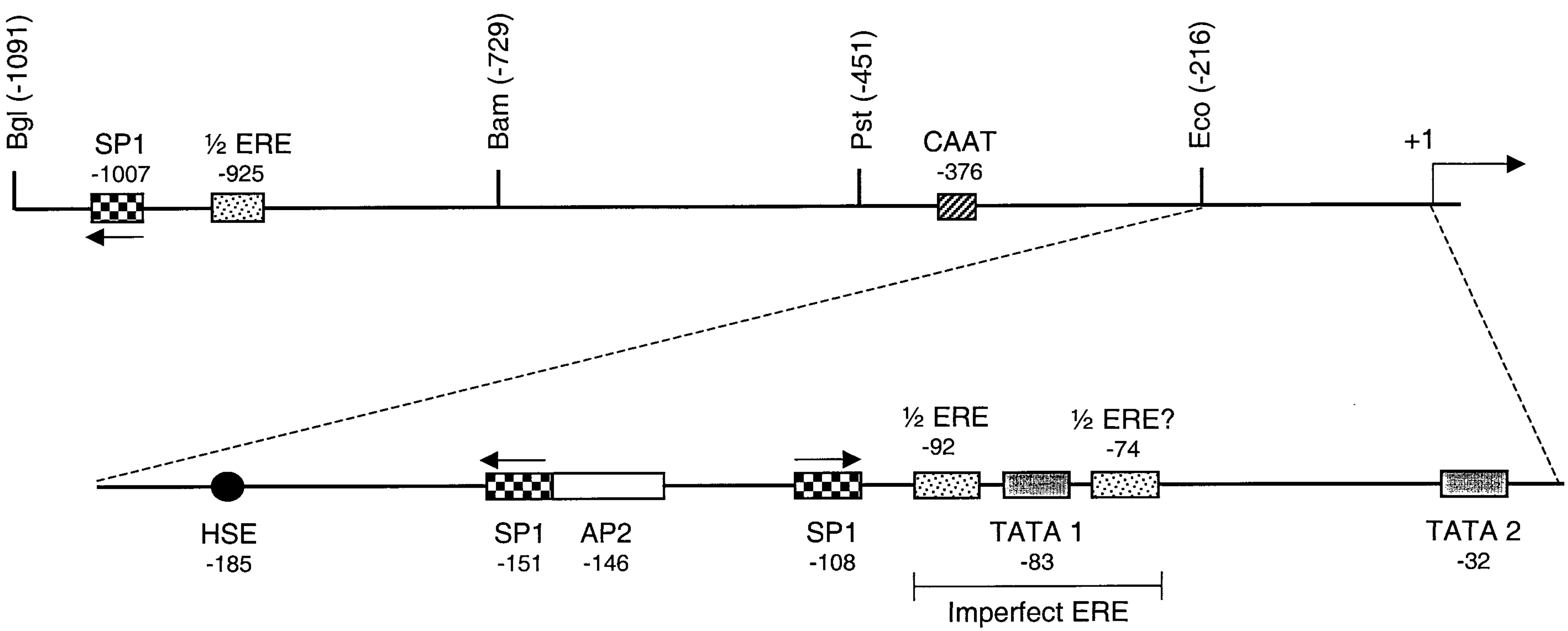

Fig. 10. *Upstream region of the* hsp27 *gene.* Top segment: 1091 bp of the *hsp27* promoter region with the proposed regulatory elements. Bottom segment: enlargement of the proximal 216 bp of the *hsp27* promoter region, TATA boxes (▩), SP1 (▦), CAAT box (▨), AP2 (☐), ERE half-site (▒), HSE (■).

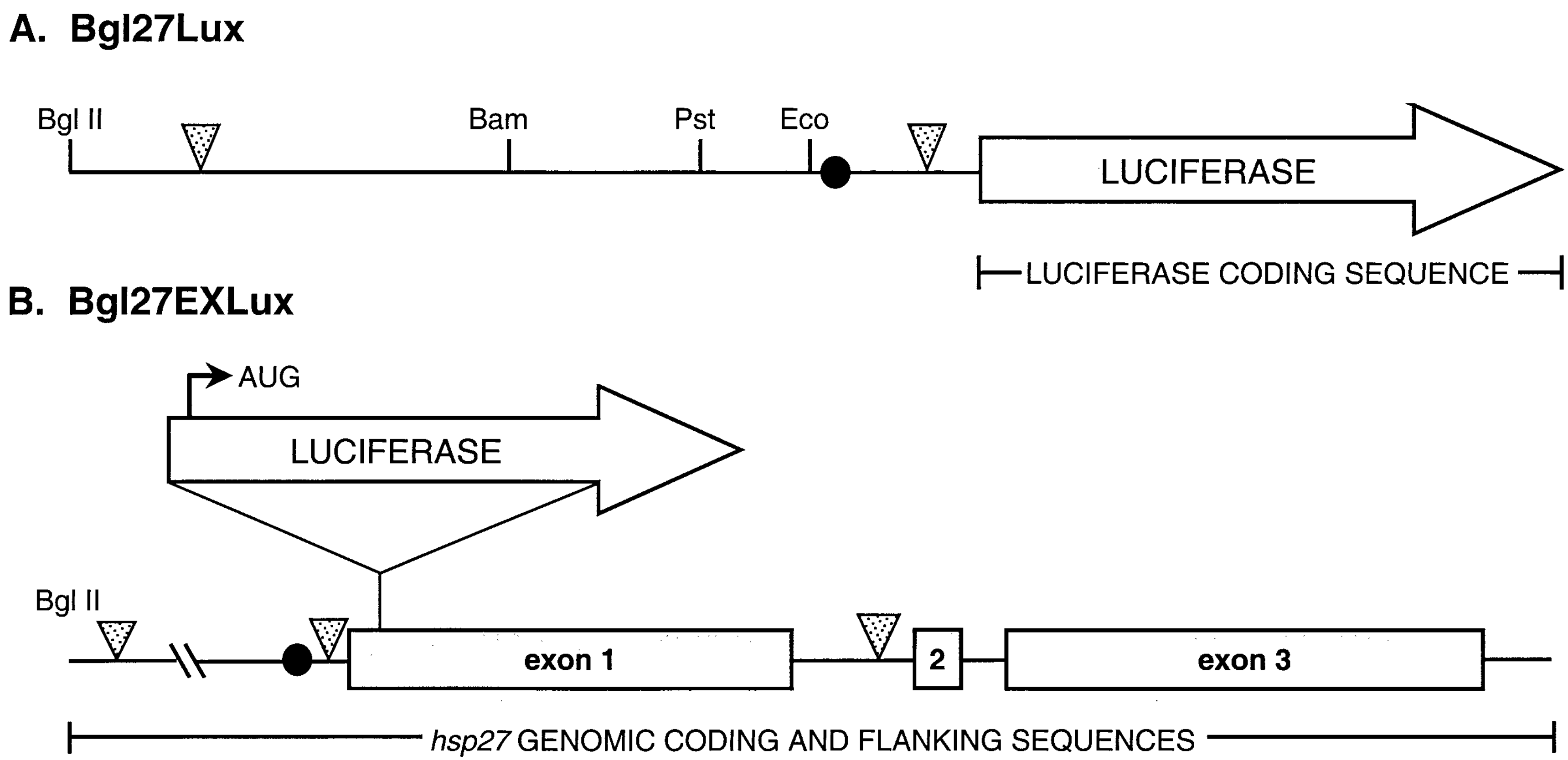

Fig. 11. *Reporter gene plasmids.* A. Bgl27Lux plasmid containing 1091 bp of *hsp27* upstream sequence driving the luciferase gene. B. Bgl27EXLux plasmid containing the *hsp27* upstream and coding region in which the luciferase gene has been placed within the first exon of the *hsp27* gene.

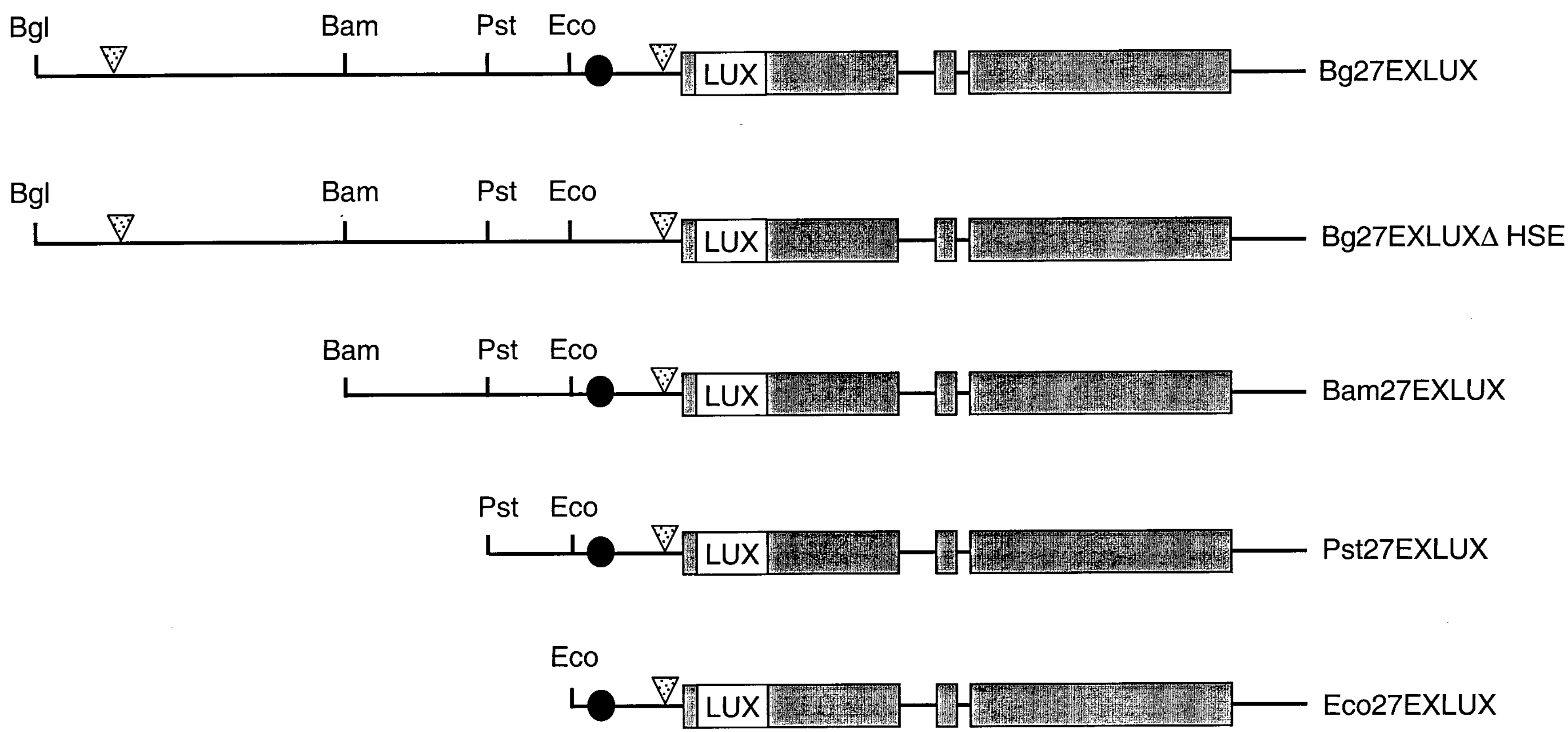

Fig. 12. *Reporter plasmids containing* hsp27 *promoter truncations.* 27EXLux plasmids containing the luciferase gene driven by truncated regions of the *hsp27* promoter sequence. Plasmids encode the following upstream segments; Bgl27EXLux 1091 bp, Bam27EXLux 729 bp, Pst27EXLux 451 bp, and Eco27EXLux 216 bp. ERE half-site (▒), HSE (■), *hsp27* coding regions (▒), luciferase (□).

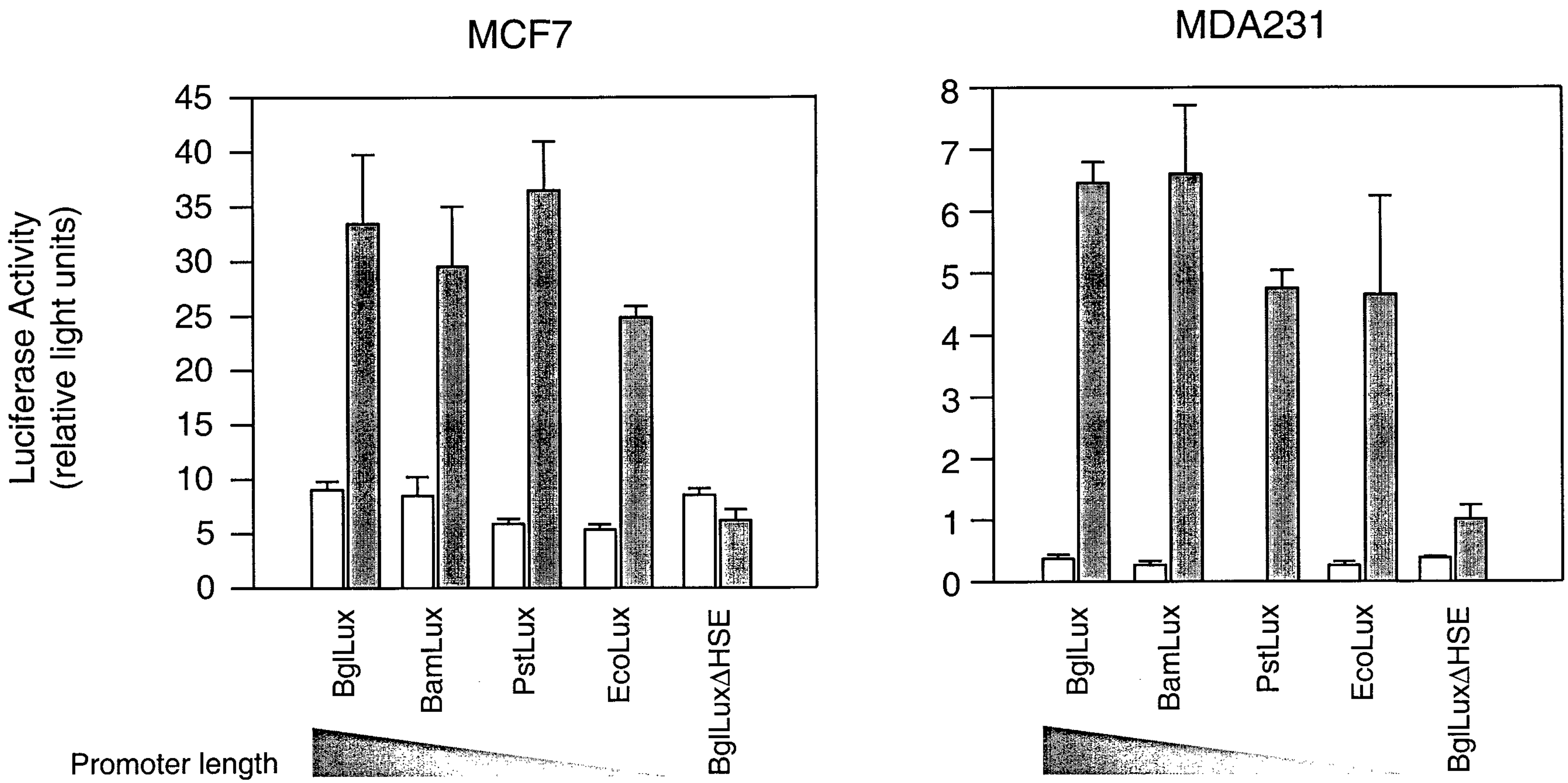

Fig. 13. *Activity of* hsp27 *truncated promoter plasmids in response to heat. hps27* reporter gene plasmids were transfected into MCF7 or MDA-231. Cells were heat treated at 42°C for 1 hour, and recovered at 37°C for 3 hours prior to lysing, or left untreated. Cell lysates were analyzed for luciferase activity; control (□), heat shock (■).

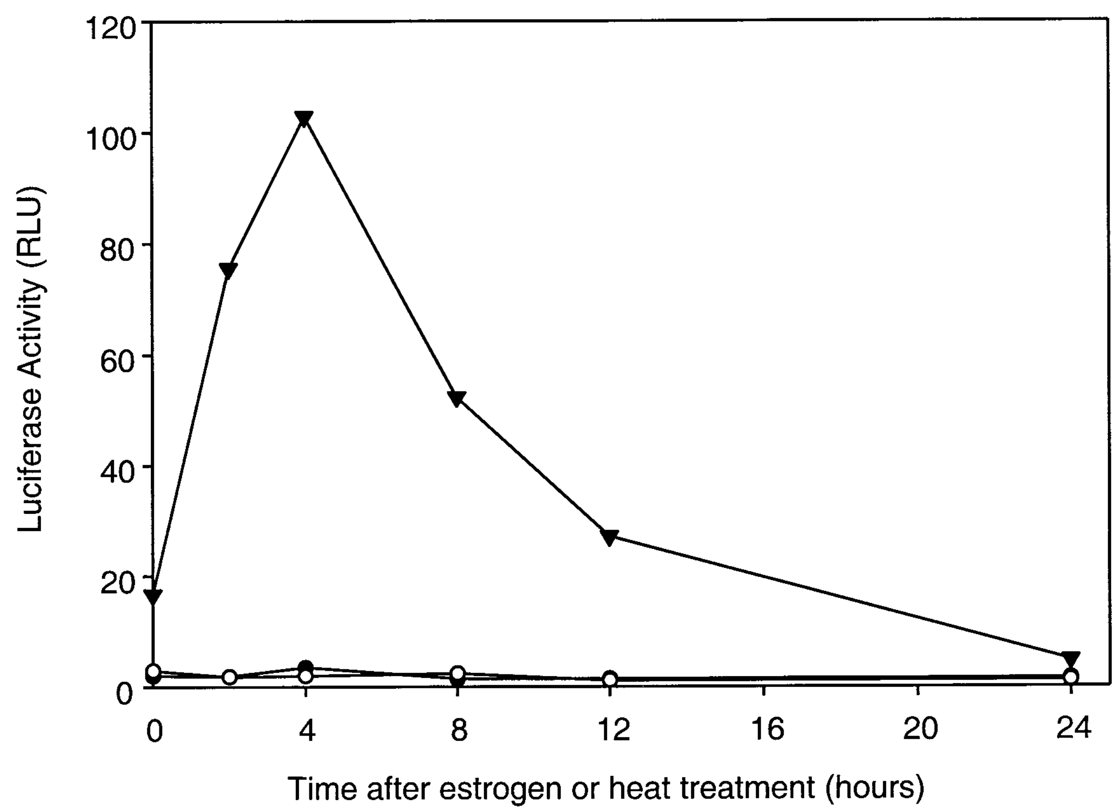

Fig. 14. *Effect of estrogen or heat treatment on* hsp27 *promoter activity.* Estrogen deprived MCF7 cells (p213) were transfected with Bgl27ExLux using Lipofectin. After 24 hours, cells were trypsinized, pooled, and replated in 6-well plates. One plate was heat treated at 42°C for 1.5 hours. A zero time point was taken immediately after heat treatment. The other cells were fed with media containing either EtOH as a control or 10^{-7} M estradiol. Cells were lysed at 0, 2, 4, 8, 12, or 24 hours after treatment, and luciferase activity was determined; control (—●—), estrogen (—○—), heat shock (—▼—).

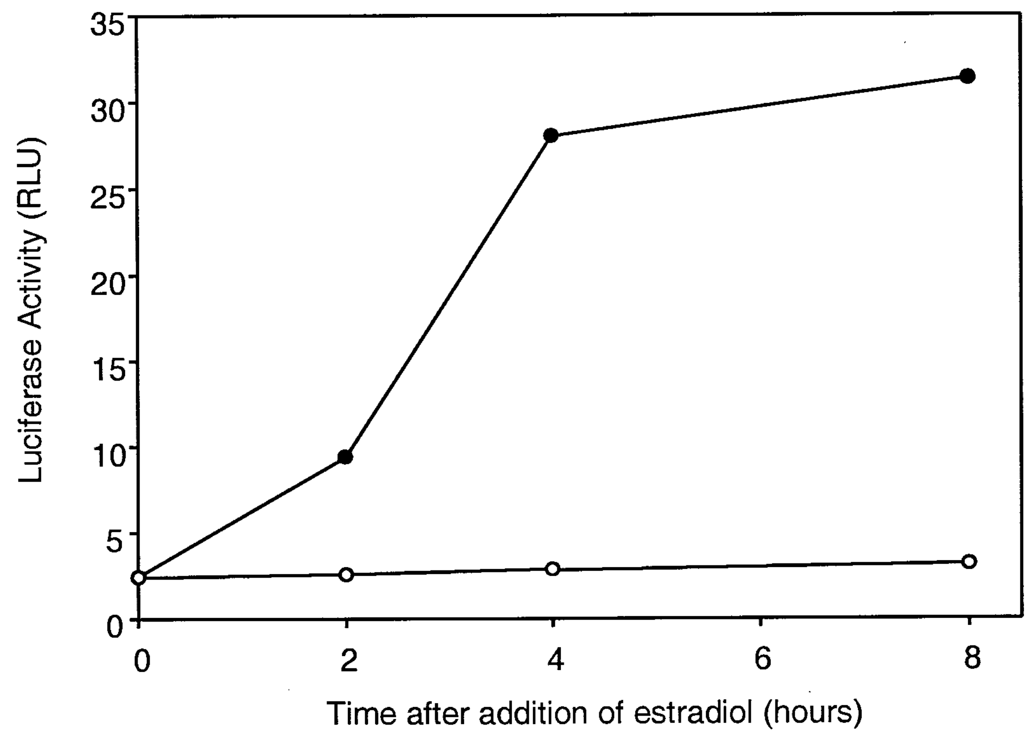

Fig. 15. *Effect of estrogen on the activity of the* hsp27 *promoter and a promoter containing a perfect ERE element.* Estrogen deprived MCF7 cells were transfected with either ERELux (—●—) or BgExLux (—○—). After 48 hours in estrogen-free media, 10^{-7} M estrogen was added, cells were lysed at the indicated times and luciferase activity was determined.

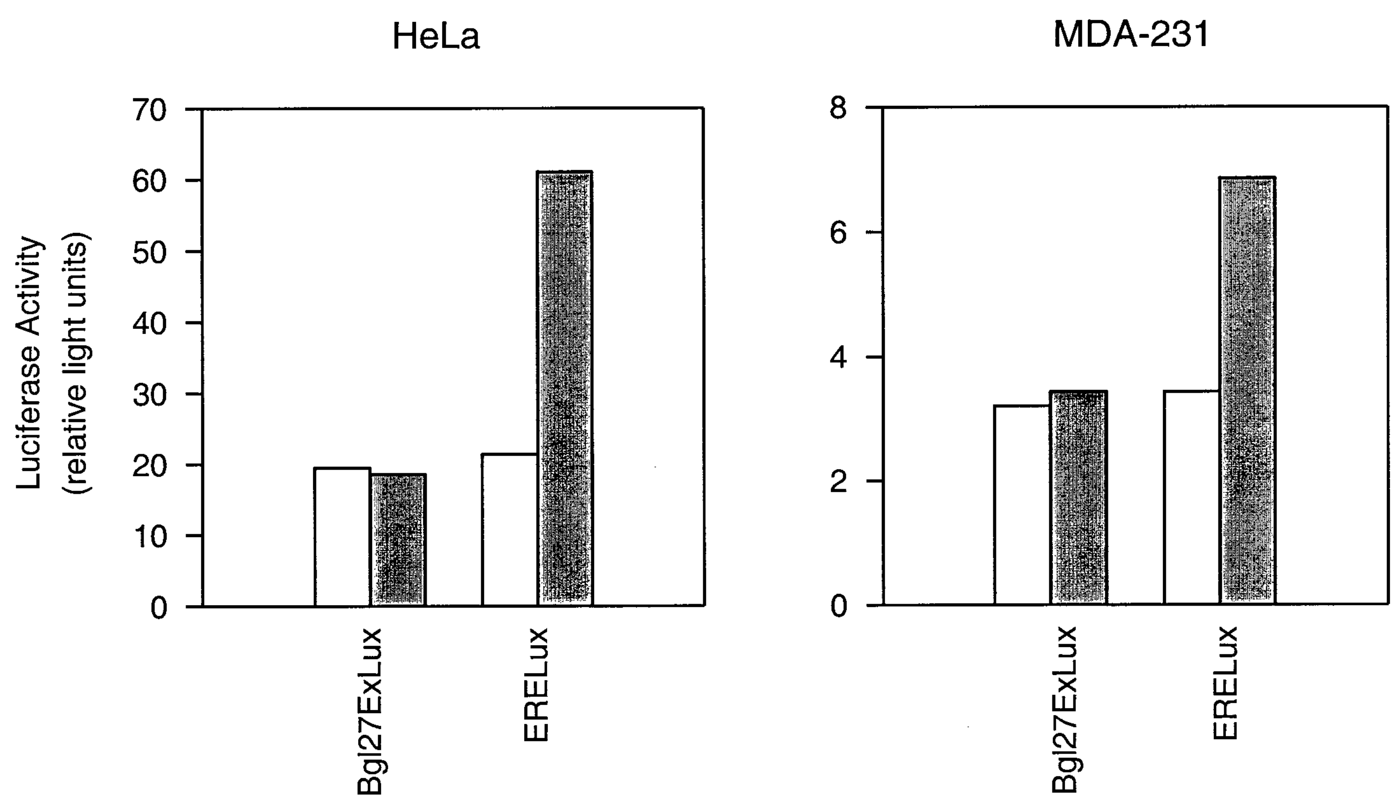

Figure 16. *Effect of transiently expressed ER on estrogen induction from Bgl27ExLux and ERELux.* MDA-231 cells were cotransfected with mutant, constitutively active ER expression construct ( ) or an empty vector, KS ( ) along with Bgl27ExLux or ERELux, and kept in regular media for 48 hours. Cells were lysed and luciferase values were determined.

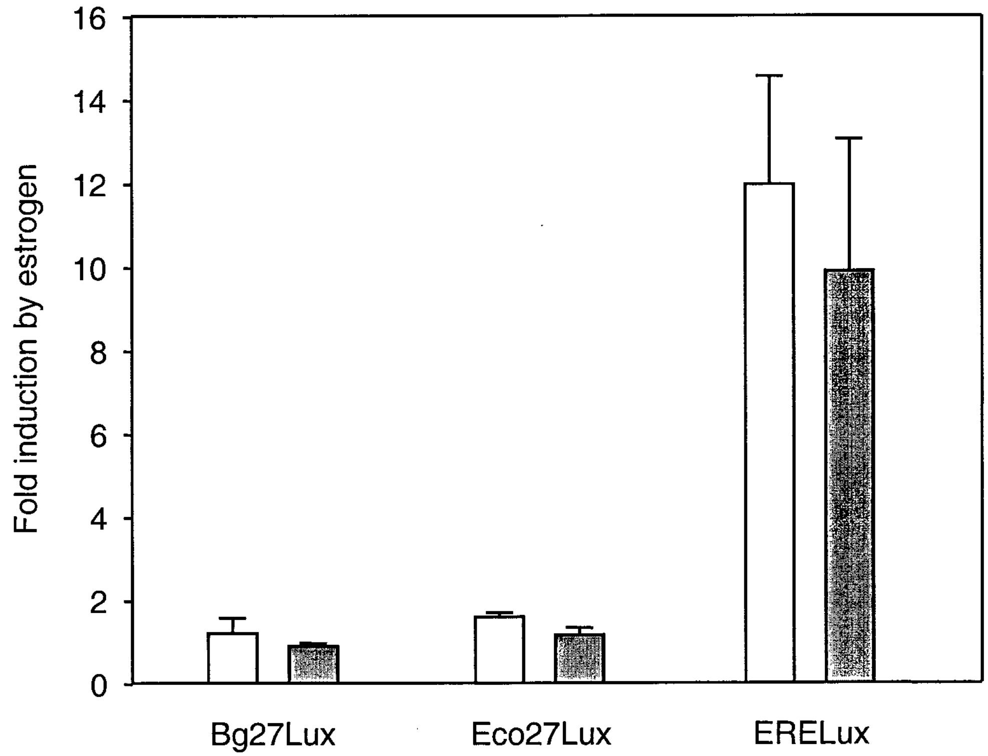

Fig. 17. *Effect of transient expression of ER on the activity of the* hsp27 *promoter or a promoter containing a perfect ERE.* Estrogen deprived MCF7 cells were cotransfected with mutant constitutively active ER or an empty vector, KS, along with ERELux, Bg27Lux, or Eco27Lux. Cells were treated with or without 10^{-7} M estrogen for 24 hours, lysed, and luciferase activity was measured. The fold induction in response to estrogen was determined and plotted; endogenous ER (□), transiently expressed ER (▩).

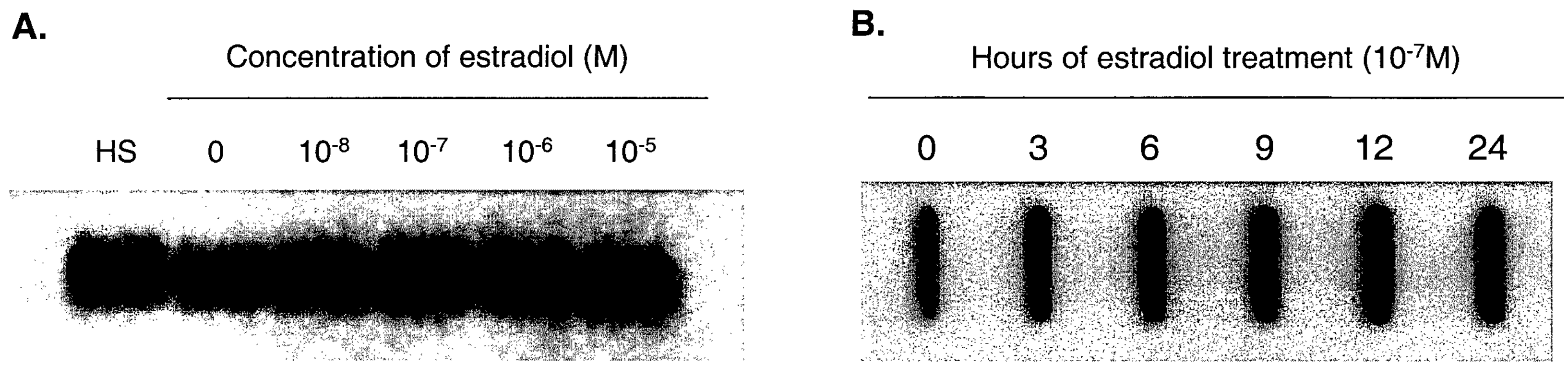

Fig. 18. *Induction of the* hsp27 *mRNA by estrogen.* A. Northern blot of *hsp27* mRNA from estrogen deprived MCF7 cells treated with the given concentration of estrogen for 24 hours (HS: heat shock). B. Slot blot of *hsp27* mRNA from estrogen depleted MCF7 cells treated with 10^{-7} M estradiol for the indicated period of time.

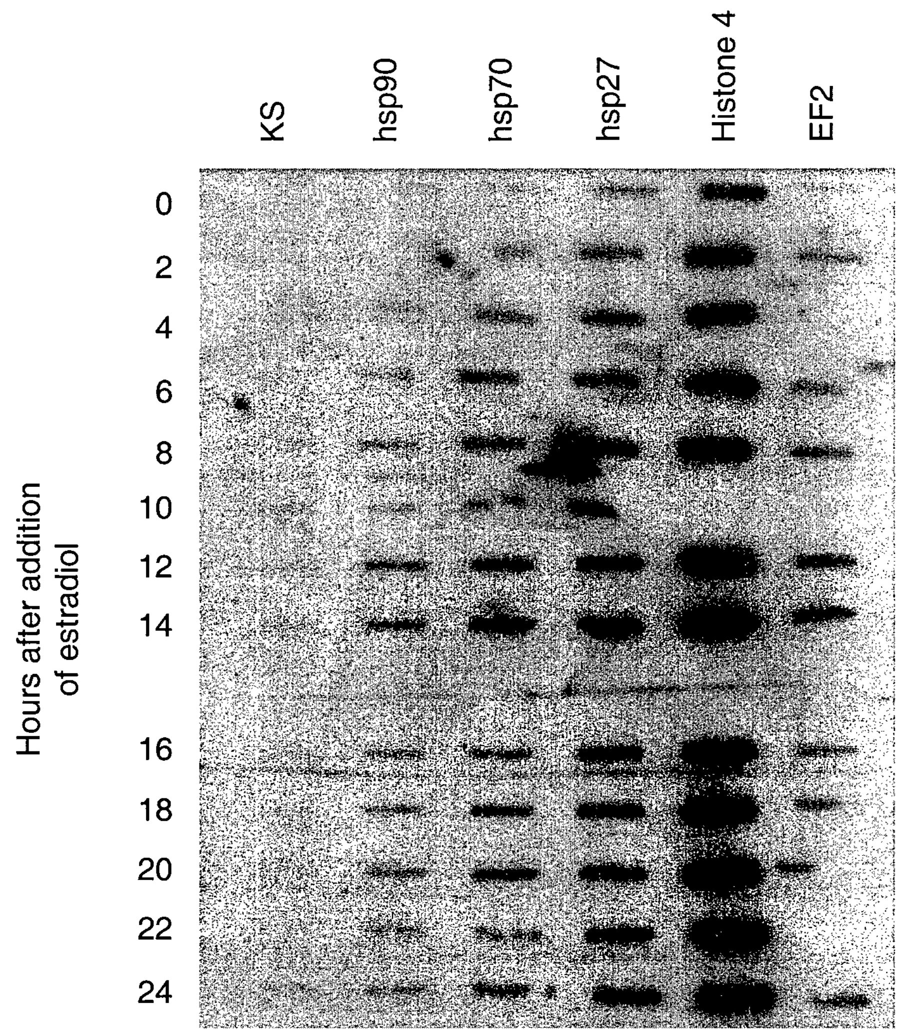

Fig. 19. *Time course of induction of nuclear mRNA transcription after addition of estradiol.* Estrogen depleted MCF7 cells were treated with 3×10^{-8} M estradiol, and nuclear fractions were extracted at the indicated times. Nuclear-run-off assays were performed on each nuclear fraction and the resulting radiolabelled cell lysates were used to probe filters previously hybridized with the indicated cDNAs.

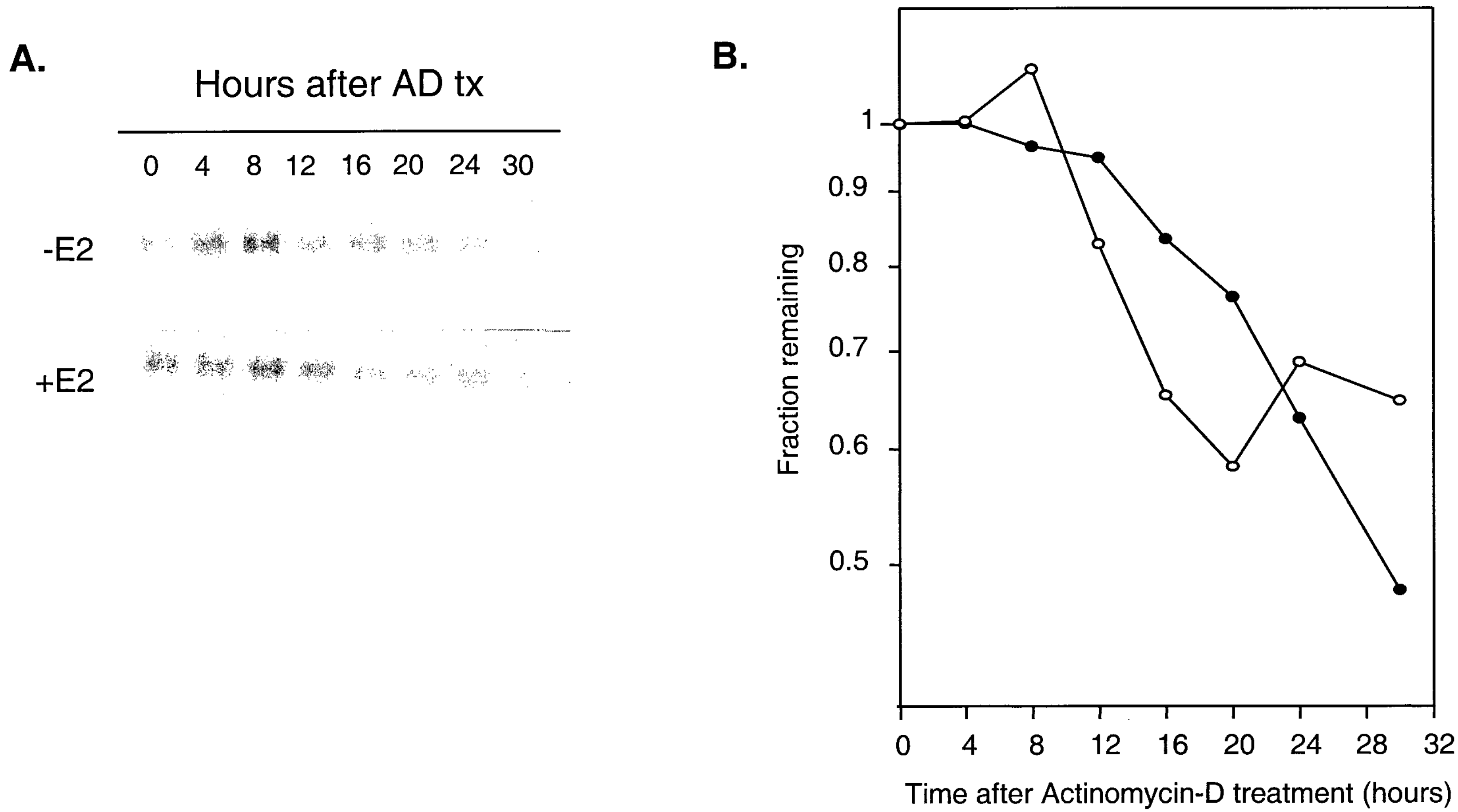

Fig. 20. *Effect of estrogen on* hsp27 *mRNA half-life.* Estrogen deprived MCF7 cells were fed with or without 10^{-7} M estrogen for 24 hours, then treated with actinomycin D (AD) at time zero. Total RNA was isolated at the indicated times after treatment with AD. A. Equal amounts of total mRNA were run on a Northern blot and *hsp27* mRNA was detected by hybridization with radiolabelled *hsp27* cDNA, and analyzed by autoradiograph. B. Quantitation of *hsp27* mRNA by densitometric analysis of the autoradiograph. The half-life determination was calculated using the time points between 12 and 30 hours. No estrogen (–●–), 10^{-7} M estrogen (–○–).